MONOCLONAL ANTIBODIES WITH REDUCED IMMUNOGENICITY

This application claims the benefit of U.S. Provisional Application No. 60/083,367, filed April 28, 1998.

Field of the Invention

This invention relates to monoclonal antibodies (mAbs) having reduced immunogenicity in humans.

Background of the Invention

Many potentially therapeutic mAbs are first generated in a murine hybridoma system for reasons of speed and simplicity. Non-human mAbs contain substantial stretches of amino acid sequences that will be immunogenic when injected into a human patient. It is well known that after injection of a foreign antibody, such as a murine antibody, a patient can have a strong human anti-mouse antibody (HAMA) response that essentially eliminates the antibody's therapeutic utility after the initial treatment as well as the utility of any other subsequently administered murine antibody.

Humanization techniques are well known for producing mAbs which exhibit reduced immunogenicity in humans while retaining the binding affinity of the original non-human parental mAb. See, *e.g.*, those disclosed in U.S. Patent Nos. 5,585,089; 5,693,761; 5,693,762; and 5,225,539.

In general, these methods depend on replacing human variable heavy and light region complementarity determining regions (CDRs) with antigen specific non-human CDRs, a process known as CDR grafting. It is also well known that in CDR grafting experiments the retention of the original antigen binding affinity is enhanced and in many cases depends on choosing human acceptor framework regions that most closely match the corresponding frameworks of the CDR donor antibody.

However, since the human genome contains a limited repertoire of heavy and light chain framework regions, these methods suffer from the limitation of available human acceptor frameworks. This restriction in acceptor framework repertoire necessarily can limit the degree of match between the non-human donor and the human acceptor antibody. Thus,

CDR grafting methods are limited by the known available repertoire of human VH and VL framework regions. Clearly, a need exists for an expanded range of acceptor V regions.

Summary of the Invention

One aspect of the present invention is an antibody comprising donor CDRs derived from an antigen-specific donor antibody of a non-human species and acceptor framework residues derived from a non-human primate.

Another aspect of the invention is a method for making an antibody having reduced immunogenicity in humans comprising grafting CDRs from antigen-specific non-human antibodies onto homologous non-human primate acceptor frameworks.

Another aspect of the invention is a chimpanzee VH acceptor framework I, II and III comprising an amino acid sequence as set forth in SEQ ID NOs: 10, 11, 12, 13, 14, 15, 16, 17 or 18.

Another aspect of the invention is a chimpanzee VH acceptor framework IV comprising an amino acid sequence as set forth in SEQ ID NOs: 81, 82, 83, 84 or 85.

Another aspect of the invention is a chimpanzee Vκ acceptor framework I, II and III comprising an amino acid sequence as set forth in SEQ ID NOs: 28, 29, 30, 31, 32, 33, 34, 35 or 36.

Another aspect of the invention is a chimpanzee Vκ acceptor framework IV comprising an amino acid sequence as set forth in SEQ ID NOs: 86 or 87.

Another aspect of the invention is a cynomolgus VH acceptor framework I, II and III comprising an amino acid sequence as set forth in SEQ ID NOs: 45, 46, 47, 48, 49, 50, 51 or 52.

Another aspect of the invention is a cynomolgus VH acceptor framework IV comprising an amino acid sequence as set forth in SEQ ID NOs: 88, 89, 90, 91, 92 or 93.

Another aspect of the invention is a cynomolgus Vκ acceptor framework I, II and III comprising an amino acid sequence as set forth in SEQ ID NOs: 59, 60, 61, 62, 63 or 64.

Another aspect of the invention is a cynomolgus Vκ acceptor framework IV comprising an amino acid sequence as set forth in SEQ ID NOs: 94, 95 or 96.

Yet another aspect of the invention is an isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NOs: 10, 11, 12, 13, 14, 15, 16, 17, 18, 28, 29, 30, 31, 32, 33, 34, 35 or 36.

Yet another aspect of the invention is an isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NOs: 81, 82, 83, 84, 85, 86 or 87.

Yet another aspect of the invention is an isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NOs: 45, 46, 47, 48, 49, 50, 51, 52, 59, 60, 61, 62, 63 or 64.

Yet another aspect of the invention is an isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NOs: 88, 89, 90, 91, 92, 93, 94, 95 or 96.

Brief Description of the Drawings

Figure 1 is an amino acid sequence of the engineered 4A6 VL region. Asterisks above the 4A6 sequence indicate the 4A6 framework residues retained in the engineered molecule. Bold and italicized letters indicate the CDRs.

Figure 2 is an amino acid sequence of the engineered 4A6 VH region. Asterisks above the 4A6 sequence indicate the 4A6 framework residues retained in the engineered molecule. Bold and italicized letters indicate the CDRs.

Figure 3 is an amino acid sequence alignment comparing the murine antibody B9Vκ with the closest matching chimpanzee Vκ and selected Jκ sequences. The CDR regions are indicated by bold and italicized letters. Gaps are indicated by dots. The numbering convention is from Kabat *et al.*, *infra*.

Figure 4 is an amino acid sequence alignment comparing the murine antibody B9VH with the closest matching chimpanzee VH and selected JH sequences. The CDR regions are indicated by bold and italicized letters. Gaps are indicated by dots. Asterisks indicate framework residues that are predicted to interact with CDRs and affect antigen binding affinity. The numbering convention is from Kabat *et al.*, *infra*.

Figure 5 is an amino acid sequence alignment comparing the murine antibody 3G9Vκ with the closest matching chimpanzee Vκ and selected Jk sequences. The CDR regions are indicated by bold and italicized letters. Gaps are indicated by dots. Asterisks indicate framework residues that are predicted to interact with CDRs and affect antigen binding affinity. The numbering convention is from Kabat *et al.*, *infra*.

Figure 6 is an amino acid sequence alignment comparing the murine antibody 3G9VH with the closest matching chimpanzee VH and selected JH sequences. The CDR regions are indicated by bold and italicized letters. Gaps are indicated by dots. Asterisks indicate framework residues that are predicted to interact with CDRs and affect antigen binding affinity. The numbering convention is from Kabat *et al.*, *infra*.

Detailed Description of the Invention

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

The molecular genetic aspects of antibody structure have been reviewed by S. Tonegawa in *Nature* 302:575-581 (1983). Briefly, antibodies are heterodimers comprised of at least two heavy and two light chains. The N-terminal domain of each heavy and light chain, termed VH and VL, respectively, fold together to form the antigen combining site. On the genetic level, the VL domain is encoded by two different gene segments, termed Vκ or Vl, and Jκ or Jl that join together to form one continuous VL region. Similarly, the VH domain is encoded by three gene segments, VH, DH, and JH, that join together to form one continuous VH region. Thus different VL and VH regions may be encoded by different combinations of Vκ or Vl, Jκ or Jl and VH, DH, and JH. This combinatorial diversity is in part the means by which the immune response generates the myriad diversity of different antibody molecules and their associated antigen specificities.

On the protein level, each heavy and light V region domain may be further divided into three CDRs. Three heavy

and three light chain CDRs fold together to form the antigen binding surface and part of the underlying support structures that are required to maintain the exact three-dimensional structure of the antigen combining site. Flanking each CDR are framework regions that in most cases do not directly interact with the specific antigen, but rather serve to form the scaffold which supports the antigen binding properties of the CDRs. Each heavy and light chain has four framework regions, three derived from the VH or VL gene segment, the fourth is derived from the JH, Jκ, or Jl gene segment. Thus, the order of frameworks and CDRs from the N- terminus is framework I, CDRI, framework II, CDRII, framework III, CDRIII, framework IV. On the genetic level, all of framework I through Framework III is encoded by the V region gene segment; CDRIII is encoded jointly by both the V region and J region gene segments; framework IV is encoded entirely from the J gene segment.

As used herein, "antibodies" refers to immunoglobulins and immunoglobulin fragments lacking all or part of an immunoglobulin constant region, *e.g.*, Fv, Fab, Fab' or $F(ab')_2$ and the like.

The term "donor antibody" refers to a monoclonal or recombinant antibody which contributes the nucleic acid sequences of its variable regions, CDRs or other functional fragments or analogs thereof to an engineered antibody, so as to provide the engineered antibody coding region and resulting expressed engineered antibody with the antigenic specificity and neutralizing activity characteristic of the donor antibody.

The term "acceptor antibody" refers to monoclonal or recombinant antibodies heterologous to the donor antibody, which contributes all, or a portion, of the nucleic acid sequences encoding its heavy and/or light chain framework regions and/or its heavy and/or light chain constant regions or V region subfamily consensus sequences to the engineered antibody.

A "functional fragment" is a partial heavy or light chain variable sequence (*e.g.*, minor deletions at the amino or carboxy terminus of the immunoglobulin variable region)

which retains the same antigen binding specificity and affinity as the antibody from which the fragment was derived.

An "analog" is an amino acid sequence modified by at least one amino acid, wherein said modification can be chemical or a substitution, which modification permits the amino acid sequence to retain the biological characteristics, *e.g.*, antigen specificity and high affinity, of the unmodified sequence.

Methods are provided for making engineered antibodies with reduced immunogenicity in humans and primates from non-human antibodies. CDRs from antigen-specific non-human antibodies, typically of rodent origin, are grafted onto homologous non-human primate acceptor frameworks. Preferably, the non-human primate acceptor frameworks are from Old World apes. Most preferably, the Old World ape acceptor framework is from *Pan troglodytes, Pan paniscus* or *Gorilla gorilla.* Particularly preferred is the chimpanzee *Pan troglodytes.* Also preferred are Old World monkey acceptor frameworks. Most preferably, the Old World monkey acceptor frameworks are from the genus *Macaca.* Particularly preferred is the cynomolgus monkey *Macaca cynomolgus.*

Particularly preferred chimpanzee (*Pan troglodytes)* heavy chain variable region frameworks (VH) are CPVH41-12 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 10 and the framework IV amino acid sequence shown in SEQ ID NO: 83; CPVH41-1 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 11 and the framework IV amino acid sequence shown in SEQ ID NO: 85; CPVH41-4 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 12; CPVH41-7 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 13; CPVH41-8 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 14, CPVH41-9 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 15 and the framework IV amino acid sequence shown in SEQ ID NO: 81; CPVH41-10 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 16 and the framework IV amino acid sequence shown in SEQ ID NO: 82; CPVH41-18 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 17; and CPVH41-19 having the framework I, II and III

amino acid sequence shown in SEQ ID NO: 18 and the framework IV amino acid sequence shown in SEQ ID NO: 84.

Particularly preferred chimpanzee (*Pan troglodytes*) light chain kappa variable region frameworks (Vκ) are CPVκ46-1 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 28; CPVκ46-3 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 29; CPVκ46-4 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 30; CPVκ46-5 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 31; CPVκ46-6 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 32 and the framework IV amino acid sequence shown in SEQ ID NO: 86; CPVκ46-7 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 33 and the framework IV amino acid sequence shown in SEQ ID NO: 87; CPVκ46-8 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 34; CPVκ46-11 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 35; and CPVκ46-14 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 36.

Particularly preferred cynomolgus (*Macaca cynomolgus*) heavy chain variable region frameworks (VH) are CYVH2-1 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 45 and the framework IV amino acid sequence shown in SEQ ID NO: 88; CYVH2-3 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 46 and the framework IV amino acid sequence shown in SEQ ID NO: 89; CYVH2-4 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 47 and the framework IV amino acid sequence shown in SEQ ID NO: 90; CYVH2-5 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 48 and the framework IV amino acid sequence shown in SEQ ID NO: 93; CYVH2-6 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 49 and the framework IV amino acid sequence shown in SEQ ID NO: 91; CYVH2-7 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 50; CYVH2-8 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 51; and CYVH2-10 having the

framework I, II and III amino acid sequence shown in SEQ ID NO: 52 and the framework IV amino acid sequence shown in SEQ ID NO: 92.

Particularly preferred cynomolgus (*Macaca cynomolgus*) light chain kappa variable region frameworks (Vκ) are CYVκ4-2 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 59; CYVκ4-3 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 60 and the framework IV amino acid sequence shown in SEQ ID NO: 94; CYVκ4-5 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 61; CYVκ4-6 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 62 and the framework IV amino acid sequence shown in SEQ ID NO: 95; CYVκ4-10 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 63; and CYVκ4-11 having the framework I, II and III amino acid sequence shown in SEQ ID NO: 64 and the framework IV amino acid sequence shown in SEQ ID NO: 96.

Isolated nucleic acid molecules encoding the chimpanzee VH and Vκ acceptor framework I, II and III amino acid sequences of SEQ ID NOs: 10, 11, 12, 13, 14, 15, 16, 17, 18, 28, 29, 30, 31, 32, 33, 34, 35 or 36 and the framework IV amino acid sequences of SEQ ID NOs: 81, 82, 83, 84 ,85, 86 or 87 are also part of the present invention. Further, isolated nucleic acid molecules encoding the cynomolgus VH and Vκ acceptor framework I, II and III amino acid sequences of SEQ ID NOs: 45, 46, 47, 48, 49, 50, 51, 52, 59, 60, 61, 62, 63 or 64 and the framework IV amino acid sequences of SEQ ID NOs: 88, 89, 90, 91, 92, 93, 94, 95 or 96 are also part of the present invention. Nucleic acid sequences encoding functional fragments or analogs of the VH and Vκ acceptor framework amino acid sequences are also part of the present invention.

In addition to isolated nucleic acid sequences encoding VH and Vk acceptor frameworks described herein, nucleic acid sequences complementary to these framework regions are also encompassed by the present invention. Useful DNA sequences include those sequences which hybridize under stringent hybridization conditions to the DNA sequences. See, T.

Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory (1982), pp. 387-389. An example of one such stringent hybridization condition is hybridization at 4XSSC at 65°C, followed by a washing in 0.1XSSC at 65°C for one hour. Alternatively, an exemplary stringent hybridization condition is 50% formamide, 4XSSC at 42°C. Preferably, these hybridizing DNA sequences are at least about 18 nucleotides in length.

Suitable frameworks are selected by computer homology searching among members of a database of Old World ape or monkey VH and VL regions. The framework portions of primate antibodies are useful as components of therapeutic antibodies. Moreover, primate antibody frameworks will be tolerated when used in the treatment of humans due to the close sequence homology between the genes of the primates and humans. Thus, the present invention provides for the grafting of CDRs from an antigen specific non-human donor antibody to acceptor V regions derived from non-human primate species.

The antigen specificity and binding kinetics of the donor antibody, which may be of rodent or any other non-human origin, are best preserved by selecting primate acceptor V regions that are determined by computer homology searching to be most similar to the donor antibody. Alternatively, the acceptor antibody may be a consensus sequence generated from primate V region subfamilies, or portions thereof, displaying the highest homology to the donor antibody.

The resulting engineered constructs, in which the donor CDRs are grafted onto primate acceptor frameworks, are subsequently refined by analysis of three-dimensional models based on known antibody crystal structures as found, *e.g.*, in the Protein Data Bank, http://www.pdb.bnl.gov/pdb-bin/pdbmain. Alternatively, computer generated three-dimensional models of the donor antibody may be computed by means of commercially available software such as "AbM" (Oxford Molecular, Oxford, UK).

Structural analysis of these models may reveal donor framework residues that are CDR-contacting residues and that are seen to be important in the presentation of CDR loops,

and therefore binding avidity. A CDR-contacting residue is one which is seen in three-dimensional models to come within the van der Waals radius of a CDR residue, or could interact with a CDR residue via a salt bridge or by hydrophobic interaction. Such donor framework (CDR-contacting) residues may be retained in the engineered construct.

The modeling experiments can also reveal which framework residues are largely exposed to the solvent environment. The engineered constructs may be further improved by substituting some or all of these solvent-accessible amino acid residues with those found at the same position among human V regions most homologous to the engineered construct as disclosed in U.S. Patent No. 5,639,641.

The engineered V regions are then joined to one or more different human or Old World ape constant regions depending on the desired secondary immune functions such as complement fixation or Fc receptor binding. Human constant regions can be selected from human immunoglobulin classes and isotypes, such as IgG (subtypes 1 through 4), IgM, IgA, and IgE. An IgG4 subtype variant containing the mutations S228P and L235E (PE mutation) in the heavy chain constant region which results in reduced effector function can also be selected. See U.S. Patent Nos. 5,624,821 and 5,648,260.

The complete heavy and light chain genes are transferred to suitable expression vectors and co-expressed in the appropriate host cells such as chinese hamster ovary, COS or myeloma cells. The resulting engineered antibody is expected to be of substantially reduced immunogenicity when administered to humans, and to retain full binding affinity for antigen.

Acceptor V regions can be isolated specifically for each donor V region by directed PCR methodology where a non-human primate cDNA library is surveyed for acceptor frameworks most similar to the donor antibody. Oligonucleotide PCR primers homologous to the donor antibody framework I (paired with C-region 3' PCR primers) are used to direct PCR amplification of a non-human primate, *e.g.*, chimpanzee lymphocyte cDNA library. This would select for V-regions with framework I regions similar to the donor antibody, and sequence analysis of the obtained clones would reveal the associated framework

II and III (and IV) sequences. 3' PCR primers would then be designed based on the knowledge of the non-human primate framework III sequences thus obtained, and used to direct PCR amplification of the original cDNA library together with a vector-specific 5' PCR primer. cDNA clones obtained from the second round of PCR amplification would have framework I and III sequences most similar to the donor antibody, and the framework II sequences would display a similar degree of sequence homology.

The present invention will now be described with reference to the following specific, non-limiting examples.

Example 1

Random cDNA Cloning and Sequence Analysis of Chimpanzee VH Regions

Five ml of peripheral blood was collected and pooled from three chimpanzees (*Pan troglodytes*) and peripheral blood mononuclear cells were isolated by standard density centrifugation methods. These cells, which include antibody producing lymphocytes, were dissolved in TRIzol reagent (GIBCO, Gaithersburg, MD, USA) and total RNA was recovered from this material by solvent extraction and precipitation according to the manufacturer's specifications.

Chimpanzee heavy chain V regions were cloned from the total RNA using Marathon RACE methodology (Clontech, Palo Alto, CA, USA) following exactly the manufacturer's protocol using 3' Cg1 gene specific primers. After RACE PCR amplification, DNA bands of the expected size were excised from agarose gels, the DNA was purified and cloned into a plasmid vector. Although this cDNA library contains many distinct heavy chain V region clones, nine were selected randomly for sequence analysis. Complete nucleic acid sequences and predicted protein sequences of the chimpanzee VH cDNA clones 41-12, 41-1, 41-4, 41-7, 41-8, 41-9, 41-10, 41-18 and 41-19 are shown in SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8 and 9, respectively. The amino acid sequences of the region from the first amino acid of the mature VH region to the second conserved cysteine residue at position 92, adjacent to CDR III of these clones, namely, CPVH41-12,

CPVH41-1, CPVH41-4, CPVH41-7, CPVH41-8, CPVH41-9, CPVH41-10, CPVH41-18 and CPVH41-19 are shown in SEQ ID NOs: 10, 11, 12, 13, 14 ,15, 16, 17 and 18, respectively. The amino acid sequence of the region encoding framework IV of these clones for CPVH41-9, CPVH41-10, CPVH41-12, CPVH41-19 and CPVH 41-1 are shown in SEQ ID NOs: 81, 82, 83, 84 and 85, respectively.

The chimpanzee VH amino acid sequences from the mature N-terminus and the second conserved cysteine residue at position 92, adjacent to CDRIII, were used as query sequences in computer homology searching of the Kabat database of Sequences of Proteins of Immunological Interest (ftp://ncbi.nlm.nih.gov/repository/kabat/) The results of this analysis are shown in Table 1.

In each case, the closest match was with a human VH region, displaying between 76% (41-1/HHC20G) and 94% (41-10/HHC20Y) sequence identity at the amino acid level. Matches were found for each of the three major human VH subgroups, indicating that the chimpanzee VH repertoire includes at least some members homologous to each of the major human subgroups. The human subgroup homology is presented in Table 1.

Table 1

Clone	Closest Match	Overall Amino Acid Homology	VH Subgroup Match
41-4	HHC10X	88%	I
41-9	HHC10Y	92	I
41-18	HHC10D	84	I
41-1	HHC20G	76	II
41-10	HHC20Y	94	II
41-12	HHC20C	83	II
41-7	HHC30T	80	III
41-8	HHC30T	79	III
41-19	HHC305	82	III

The results show that the overall sequence identity between the chimpanzee and human VH regions ranged between 76 and 95% with a mean identity of 84%. Based on this observation, further sampling of the chimpanzee random VH library will likely provide a substantially greater diversity of VH sequences from which to choose optimum acceptor frameworks for each particular donor VH region.

Example 2

Random cDNA Cloning and Sequence Analysis of Chimpanzee Vκ Regions

Chimpanzee light chain Vκ regions were cloned from the total RNA using Marathon RACE methodology (Clontech, Palo Alto, CA, USA) following exactly the manufacturer's protocol and Cκ 3' gene specific primers. After RACE PCR amplification, DNA bands of the expected size were excised from agarose gels, the DNA was purified and cloned into a plasmid vector. Although this cDNA library contains many distinct light chain Vκ region clones, nine were selected randomly for sequence analysis. Complete nucleic acid sequences and predicted protein sequences of the chimpanzee Vκ cDNA clones 46-1, 46-3, 46-4, 46-5, 46-6, 46-7, 46-8, 46-11 and 46-14 are shown in SEQ ID NOs: 19, 20, 21, 22, 23, 24, 25, 26 and 27, respectively. The amino acid sequences of the region from the first amino acid of the mature Vκ region to the second conserved cysteine residue at position 88, adjacent to CDR III of these clones, namely CPVκ46-1, CPVκ46-3, CPVκ46-4, CPVκ46-5, CPVκ46-6, CPVκ46-7, CPVκ46-8, CPVκ46-11 and CPVκ46-14 are shown in SEQ ID NOs: 28, 29, 30, 31, 32, 33, 34, 35 and 36, respectively. The amino acid sequences of the region encoding framework IV of these clones for CPVκ46-6 and CPVκ46-7 are shown in SEQ ID NOs: 86 and 87, respectively.

The chimpanzee Vκ amino acid sequences comprising the mature N-terminus and the second conserved cysteine residue at position 88 were used as query sequences in computer homology searching of the Kabat database. The results of this analysis are shown in Table 2. In each case the closest match was with a human Vκ region, displaying between 68% (46-4/HKL310) and 97% (46-11/HKL106) sequence identity at the amino acid level. It is evident that the chimpanzee Vκ sequences are distinct from the collection of human Vκ found in the Kabat database.

The human subgroup homology is presented in Table 2. Of the four major human Vκ subgroups, matches were found for the two most frequently isolated, indicating that the chimpanzee Vκ repertoire is at least homologous to members of the majority of the human Vκ repertoire. Further sampling of the chimpanzee Vκ cDNA library will likely identify a greater diversity of chimpanzee Vκ regions, including ones homologous to the remaining two human Vκ subgroups (VκII and VκIV).

Table 2

Clone	Closest Match	Overall Amino Acid Homology	VH Subgroup Match
46-1	HKL10C	85%	I
46-3	HKL 10O	91	I
46-5	HKL 10O	91	I
46-7	HKL 10O	81	I
46-8	HKL 10N	90	I
46-11	HKL 106	97	I
46-14	HKL 10O	92	I
46-4	HKL 310	68	III
46-6	HKL 310	96	III

Example 3

Random cDNA Cloning and Sequence Analysis of Cynomolgus VH Regions

Splenic RNA was recovered from a single donor cynomolgus monkey (*Macaca cynomolgus*) by means of standard laboratory practice. Cynomolgus heavy chain V regions were cloned from the total RNA using Marathon RACE methodology (Clontech, Palo Alto, CA, USA) following exactly the manufacturer's protocol using 3' Cg1 gene specific primers. After RACE PCR amplification, DNA bands of the expected size were excised from agarose gels, the DNA was purified and cloned into a plasmid vector. Although this cDNA library contains many distinct heavy V region clones, eight were selected randomly for sequence analysis. Complete nucleic acid sequences and predicted protein sequences of the Cynomolgus VH cDNA clones 2-1, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8 and 2-10 are shown in SEQ ID NOs: 37, 38, 39, 40, 41, 42, 43 and 44, respectively. The amino acid sequences of the region from the first amino acid of the mature VH region to the second conserved cysteine residue at position 92, adjacent to CDR III of these clones, namely CyVH2-1, CyVH2-3, CyVH2-4, CyVH2-5, CyVH2-6, CyVH2-7, CyVH2-8 and CyVH2-10 are shown in SEQ ID NOs: 45, 46, 47, 48,

49, 50, 51 and 52, respectively. The amino acid sequences of the region encoding framework IV of these clones for CyVH2-1, CyVH2-3, CyVH2-4, CyVH2-6, CyVH2-10 and CyVH2-5 are shown in SEQ ID NOs: 88, 89, 90, 91, 92 and 93, respectively.

The cynomolgus VH amino acid sequences from the mature N-terminus and the second conserved cysteine residue at position 92, adjacent to CDRIII, were used as query sequences in computer homology searching of the Kabat database. The results of this analysis are shown in Table 3. In each case the closest match was with a human VH region, displaying between 62% (2-6/ HHC20E) and 84% (2-5/ HHC20F) sequence identity at the amino acid level. It is evident that the cynomolgus VH sequences are distinct from the collection of human VH found in the Kabat database. Matches were found for each of the three major human VH subgroups, indicating that the cynomolgus VH repertoire includes at least some members homologous to each of the major human subgroups. The human subgroup homology is presented in Table 3.

Table 3

Clone	Closest Match	Overall Amino Acid Homology	VH Subgroup Match
2-4	HHC10Y	83%	I
2-10	HHC20G	83	II
2-8	HHC20F	74	II
2-6	HHC20E	62	II
2-5	HHC20F	84	II
2-3	HHC20F	75	II
2-1	HHC316	71	III
2-7	HHC31C	81	III

The results show that the overall sequence identity between the cynomolgus and human VH regions ranged between 62 and 84% with a mean identity of 77%. Based on this observation, further sampling of the cynomolgus random VH library will likely provide a substantially greater diversity of VH sequences from which to choose optimum acceptor frameworks for each particular donor VH region.

Example 4

Random cDNA Cloning and Sequence Analysis of Cynomolgus Vκ Regions

Cynomolgus light chain Vκ regions were cloned from the total splenic RNA using Marathon RACE methodology (Clontech,

Palo Alto, CA, USA) following exactly the manufacturer's protocol and Cκ 3' gene specific primers. After RACE PCR amplification, DNA bands of the expected size were excised from agarose gels, the DNA was purified and cloned into a plasmid vector. Although this cDNA library contains many distinct light chain Vκ region clones, six were selected randomly for sequence analysis. Complete nucleic acid sequences and predicted protein sequences of the Cynomolgus Vκ cDNA clones 4-2, 4-3, 4-5, 4-6, 4-10 and 4-11 are shown in SEQ ID NOs: 53, 54, 55, 56, 57 and 58, respectively. The amino acid sequences of the region from the first amino acid of the mature Vκ region to the second conserved cysteine residue at position 88, adjacent to CDRIII, of these clones, namely CyVκ4-2, CyVκ4-3, CyVκ4-5, CyVκ4-6, CyVκ4-10 and CyVκ4-11 are shown in SEQ ID NOs: 59, 60, 61, 62, 63 and 64, respectively. The amino acid sequences encoding the framework IV region of these clones for CyVκ4-3, CyVκ4-6 and CyVκ4-11 are shown in SEQ ID NOs: 94, 95 and 96, respectively.

The cynomolgus Vκ amino acid sequences comprising the mature N-terminus and the second conserved cysteine residue at position 88 were used as query sequences in computer homology searching of the Kabat database. The results of this analysis are shown in Table 4. In each case the closest match was with a human Vκ region, displaying between 73% (4-11/ HKL10S) and 94% (4-3/ HKL400) sequence identity at the amino acid level. It is evident that the cynomolgus Vκ sequences are distinct from the collection of human Vκ found in the public genetic databases. The human subgroup homology is presented in Table 4. Matches were found for three of the four major human Vk subgroups, indicating that the cynomolgus Vκ repertoire is largely homologous to members of the majority of the human Vκ repertoire. Further sampling of the cynomolgus Vκ cDNA library will likely identify a greater diversity of cynomolgus Vκ regions, including ones homologous to the remaining human Vκ subgroup (VκIII).

Table 4

Clone	Closest Match	Overall Amino Acid Homology	Vκ Subgroup Match
4-6	HKL10L	80%	I
4-2	HKL10Z	83	I
4-11	HKL10S	73	I
4-10	HKL10F	93	I
4-5	HKL209	86	II
4-3	HKL400	94	IV

The results show that the overall sequence identity between the cynomolgus and human Vκ regions ranged between 73 and 94% with a mean identity of 85%. Based on this observation, further sampling of the cynomolgus random Vκ library will provide a substantially greater diversity of Vκ sequences from which to choose optimum acceptor frameworks for each particular donor Vκ region.

Example 5

Preparation of Engineered Anti-IL-5 Monoclonal Antibodies

The Vκ and VH genes of the rat anti-interleukin-5 (IL-5) antibody 4A6 are shown in SEQ ID NOs: 65 and 66, respectively. These genes encode a high affinity neutralizing monoclonal antibody specific for human IL-5 useful for the treatment of asthma. See U.S. Patent No. 5,693,323.

The 4A6 light chain was engineered as follows. The sequence of donor antibody Vκ4A6 (SEQ ID NO: 65) was aligned with the acceptor antibody light chain Vκ region from the chimpanzee Mab C108G (*Mol. Immunol. 32*:1081-1092 (1995)) (SEQ ID NO: 67) as shown in Fig. 1. Since native Vκ4A6 has a unique deletion of residue 10, the sequence alignment included the insertion of a gap at that position. The CDR residues were identified as defined by the convention of Kabat *et al.* in *Sequences of Proteins of Immunological Interest,* 4th ed., U.S. Department of Health and Human Services, National Institutes of Health (1987).

Framework residues that could influence CDR presentation were identified by analysis of three-dimensional models based on known antibody crystal structures. The residues of this

CDR-contacting set were compared among the aligned VK4A6 and VKC108G sequences, and the positions of the set that differed between the VK4A6 and the VKC108G were marked (Fig. 1, asterisks). The CDRs and the marked framework residues of VK4A6 (the donor antibody) were transferred replacing the corresponding residues of VKC108G (the acceptor antibody). The completed engineered 4A6 light chain V region is shown in SEQ ID NO: 68. Six donor framework residues were retained in the engineered molecule at residues 1 to 4, 49 and 60.

In analogous fashion, a similar method was used to engineer the 4A6 heavy chain. The sequence of donor antibody VH4A6 (SEQ ID NO: 66) was aligned with the acceptor antibody heavy chain V region from the chimpanzee Mab C108G (SEQ ID NO: 69) as shown in Fig. 2. A large gap was introduced in the VH4A6 CDRIII alignment, as CDRIII of VHC108G is 10 residues longer. CDR residues were identified as defined by the convention of Kabat *et al.*, *supra*.

Framework residues that could influence CDR presentation were identified by analysis of three-dimensional models based on known antibody crystal structures. The residues of this CDR-contacting set were compared among the aligned VH4A6 and VHC108G sequences, and the positions of the set that differed between the VH4A6 and the VHC108G were marked (Fig. 2, asterisks). In total, 11 such CDR contacting residues that differed between VH4A6 and the VHC108G were selected and marked. The CDRs and the marked CDR contacting framework residues of VH4A6 (the donor antibody) were transferred replacing the corresponding residues of VHC108G (the acceptor antibody). The completed engineered 4A6 heavy chain V region is shown in SEQ ID NO: 70. Eleven donor framework residues were retained in the engineered molecule at residues 27, 30, 38, 49, 66, 67, 69, 71, 73, 78 and 94.

The engineered 4A6 can be expressed in cells using methods well known to those skilled in the art. Briefly, genes encoding the complete engineered 4A6 VH and VK regions can be assembled from long synthetic oligonucleotides and ligated into appropriate eukaryotic expression vectors containing the desired antibody constant regions. Such an expression vector will contain selectable markers, for

example, neomycin resistance and regulatory sequences, for example, the CMV promoter, required to direct the expression of full-length antibody heavy and light chains. Subsequently, transfection of the appropriate host cell, for example, chinese hamster ovary, would result in the expression of fully active engineered 4A6.

Example 6

Preparation of Engineered Anti-Integrin Monoclonal Antibodies

The Vκ and VH genes of the murine anti-integrin antibody B9 are shown in SEQ ID NOs: 71 and 72, respectively. These genes encode a high affinity neutralizing monoclonal antibody specific for human integrin αvβ3 useful for the treatment of vascular diseases.

The B9 light chain was engineered as follows. The amino acid sequence of donor antibody VκB9 (SEQ ID NO: 72) was compared to each of the nine chimpanzee Vκ sequences described above and percent sequence identity determined by computer homology searching using the LASERGENE program "MEGALIGN" (DNASTAR, Inc., Madison, WI). Clones CPVκ46-3 (SEQ ID NO: 29) and CPVκ46-14 (SEQ ID NO: 36) were identified as the chimpanzee Vκ regions with the highest overall sequence similarity (77%) to the B9 donor Vκ. CPVκ46-3 was selected as the acceptor framework.

Similarly, the chimpanzee Jκ gene segment of CPVκ46-1 (SEQ ID NO: 97) was selected as acceptor framework IV. The sequences of the donor VκB9 and acceptor CPVκ46-3, CPVκ46-1 V regions were aligned and the positions of their respective framework and CDRs were determined as shown in Fig. 3.

The CDR residues were identified as defined by the convention of Kabat *et al.*, *supra*. The results show that VκB9 and CPVκ46-3 share 77% overall sequence identity, with the framework regions I through III sharing 81% sequence identity.

Framework residues that could influence CDR presentation were identified by analysis of three-dimensional models based on known antibody crystal structures. The residues of this

CDR-contacting set were compared among the aligned VkB9 and CPVk46-3 sequences, and none of this set were found that differed between the VkB9 and the CPVk46-3. Accordingly, only the CDRs of VkB9 (the donor antibody) were transferred replacing the corresponding residues of CPVk46-3 (the acceptor antibody). Lastly, the framework IV sequences of CPVk46-1 replaced the corresponding framework IV residues of the B9 light chain variable region. The completed engineered B9 light chain V region is shown in SEQ ID NO: 73. No donor framework residues were retained in the engineered light chain variable region.

The B9 heavy chain was engineered in analogous fashion. The amino acid sequence of donor antibody VHB9 (SEQ ID NO: 71) was compared to each of the nine chimpanzee VH sequences described above by computer homology searching. Clone CPVH41-18 (SEQ ID NO: 17) was identified as the chimpanzee VH region with the highest overall sequence similarity (58%) to the B9 donor VH.

The chimpanzee JH gene segment of CPVH41-10 (SEQ ID NO: 82) was selected as acceptor framework IV. The sequences of the donor VHB9 and chimpanzee acceptor V regions were aligned and the positions of their respective framework and CDRs determined as shown in Fig. 4.

The CDR residues were identified as defined by the convention of Kabat *et al., supra*. The results show that VHB9 and CPVH41-18 share 58% overall sequence identity, with the framework regions I through III sharing 65% sequence identity.

Framework residues that could influence CDR presentation were identified by analysis of three-dimensional models based on known antibody crystal structures. The residues of this CDR-contacting set were compared among the aligned VHB9 and CPVH41-18 sequences, and the nine residues of the set that differed between VHB9 and the chimpanzee acceptor frameworks were marked. The CDRs and the marked framework residues of donor antibody VHB9 were transferred replacing the corresponding residues of CPVH41-18 (the acceptor antibody). Lastly, the framework IV sequences of CPVH41-10 replaced the corresponding framework IV residues of the B9 heavy chain variable region. The completed engineered B9 heavy chain V

region is shown in SEQ ID NO: 74. Nine donor framework residues were retained in the engineered heavy chain variable region at positions 24, 27, 38, 48, 66, 67, 69, 93 and 94.

Example 7

Expression and Characterization of Engineered Anti-Integrin Monoclonal Antibodies

The engineered B9 antibody was expressed in cells using methods well known to those skilled in the art. Briefly, genes encoding the complete engineered B9 VH and Vκ regions were assembled from long synthetic oligonucleotides and ligated into appropriate eukaryotic expression vectors containing IgG1,κ antibody constant regions. The expression vector contained a selectable marker for neomycin resistance and CMV promoter regulatory sequences. Subsequent transfection of a COS host cell resulted in the expression of engineered B9 (CPB9).

The relative binding avidity of CPB9 was compared to that of the original murine B9 antibody as follows. CPB9 antibodies present in culture supernatants from cells maintained in culture for 5 days after transfection with the expression constructs were compared to the parental murine B9 antibody using the ORIGEN technology (IGEN Inc, Gaithersburg, MD). Briefly, different dilutions of the B9 variants were incubated with purified human αvβ3 integrin which had previously been biotinylated, and an electrochemiluminescent TAG moiety specific for the antibody C regions. B9 variant antibody bound to the integrin was measured by capturing the immune complexes onto streptavidin beads followed by analysis on the ORIGEN instrument. The results showed that the CPB9 and the murine B9 binding curves were displaced only by about 3-fold indicating that the overall specific binding avidity of CPB9 and murine B9 for αvβ3 are within three-fold of each other. Accordingly, the results show that the CDR grafting of rodent CDRs onto chimpanzee frameworks as described in the present invention retained nearly all of the binding avidity of the parent rodent mAb.

Example 8

Preparation of Engineered Anti-Erythropoietin Receptor Monoclonal Antibodies

The VH and Vκ genes of the murine anti-erythropoietin receptor antibody 3G9 are shown in SEQ ID NOs: 75 and 76, respectively. These genes encode a high affinity neutralizing monoclonal antibody specific for human erythropoietin receptor (EPOr) useful for the treatment of hematopoietic disorders.

The 3G9 light chain was engineered as follows. The amino acid sequence of donor antibody Vκ3G9 (SEQ ID NO: 76) was compared to each of the nine chimpanzee Vκ sequences described above by computer homology searching as described above. Clones CPVκ46-3 (SEQ ID NO: 29), CPVκ46-5 (SEQ ID NO: 31), CPVκ46-8 (SEQ ID NO: 34) and CPVκ46-14 (SEQ ID NO: 36) were identified as the chimpanzee Vκ regions with the highest overall sequence similarity (65%) to the 3G9 donor Vκ. CPVκ46-14 was selected as the acceptor framework.

The chimpanzee Jκ gene segment of CPVκ46-14 was identical to that of CPVκ46-1 (SEQ ID NO: 97) and was selected as acceptor framework IV. The sequences of the donor Vκ3G9 and acceptor CPVκ46-14 V regions were aligned and the positions of their respective framework and CDRs were determined as shown in Fig. 5.

The CDR residues were identified as defined by the convention of Kabat *et al.*, *supra*. The results show that Vκ3G9 and CPVκ46-14 share 65% overall sequence identity, with the framework regions I through III sharing 73% sequence identity.

Framework residues that could influence CDR presentation were identified by analysis of three-dimensional models based on known antibody crystal structures. The residues of this CDR-contacting set were compared among the aligned Vκ3G9 and CPVκ46-14 sequences, and the positions of this set that differed between Vκ3G9 and the CPVκ46-3 were marked. The CDRs and marked residues of Vκ3G9 (the donor antibody) were

transferred replacing the corresponding residues of CPVκ46-14 (the acceptor antibody). Lastly, the framework IV sequences of CPVκ46-14 replaced the corresponding framework IV residues of the 3G9 light chain variable region. The completed engineered 3G9 light chain V region is shown in SEQ ID NO: 77. Three donor framework residues were retained in the engineered light chain variable region at positions 3, 46 and 60.

The 3G9 heavy chain was engineered in analogous fashion. The amino acid sequence of donor antibody VH3G9 (SEQ ID NO: 75) was compared to each of the 9 chimpanzee VH sequences described above by computer homology searching. Clone CPVH41-18 (SEQ ID NO: 17) was identified as the chimpanzee VH region with the highest overall sequence similarity (53%) to the 3G9 donor VH.

The chimpanzee JH gene segment of CPVH41-18 was identical to CPVH41-9 (SEQ ID NO: 81) and was selected as acceptor framework IV. The sequences of the donor VH3G9 and chimpanzee acceptor V regions were aligned and the positions of their respective framework and CDRs determined as shown in Fig. 6.

The CDR residues were identified as defined by the convention of Kabat *et al.*, *supra*. The results show that VH3G9 and CPVH41-18 share 53% overall sequence identity, with the framework regions I through III sharing 62% sequence identity.

Framework residues that could influence CDR presentation were identified by analysis of three-dimensional models based on known antibody crystal structures. The residues of this CDR-contacting set were compared among the aligned VH3G9 and CPVH41-18 sequences, and the twelve residues of the set that differed between VH3G9 and the chimpanzee acceptor frameworks were marked. The CDRs and the marked framework residues of donor antibody VH3G9 were transferred replacing the corresponding residues of CPVH41-18 (the acceptor antibody). Lastly, the framework IV sequences of CPVH41-18 replaced the corresponding framework IV residues of the 3G9 heavy chain variable region. The completed engineered 3G9 heavy chain V region is shown in SEQ ID NO: 78. Twelve donor framework residues were retained in the engineered heavy chain variable

region at positions 24, 27, 30, 38, 48, 66-69, 71, 73, and 94.

Example 9

Expression and Characterization of Engineered anti-Erythropoietin Receptor Monoclonal Antibodies

The engineered 3G9 antibody was expressed in cells using methods well known to those skilled in the art. Briefly, genes encoding the complete engineered 3G9 VH and Vκ regions were assembled from long synthetic oligonucleotides and ligated into appropriate eukaryotic expression vectors containing IgG1,κ antibody constant regions. The expression vector contained a selectable marker for neomycin resistance and CMV promoter regulatory sequences. Subsequent transfection of COS host cells resulted in the expression of engineered 3G9 (CP3G9).

Culture supernatants from COS cells transiently transfected with chimpanzee framework engineered 3G9 were compared with another 3G9 variant for the ability to bind human EPOr. The entire extracellular domain of the EPOr was expressed as recombinant protein, purified, and adsorbed onto the wells of ELISA plates. Dilutions of different antibodies were then tested for the ability to specifically bind to the solid phase associated EPOr.

HZ3G9 is a humanized variant of 3G9 in which human frameworks were used in traditional CDR grafting experiments. The humanized 3G9 heavy chain amino acid sequence is shown in SEQ ID NO: 79. The humanized 3G9 light chain sequence is shown in SEQ ID NO: 80. Previous experiments showed that HZ3G9 retained the full binding affinity and avidity of the parental murine 3G9. Accordingly, since HZ3G9G1 is identical to the chimpanzee version in all respects except the V region cassette, it was used in the present comparative binding experiments as a surrogate for murine 3G9. Negative control antibodies were also tested, including HZD12 which is a humanized antibody specific for human integrin, and CPB9 which is a chimpanzee framework engineered antibody specific for human integrins described above. Different concentrations of the 3G9 variants and control antibodies were incubated for one hour. After washing, the bound

antibodies were detected by incubation with anti-human H+L antibody-enzyme conjugate, a final wash, and addition of chromagen.

The binding curves obtained for CP3G9 and HZ3G9 were superimposable. This result indicates that the human and the chimpanzee framework engineered versions of 3G9 have identical overall binding avidity for the specific antigen human EPOr. Since the constant regions of HZ3G9 and CP3G9 are identical, the results also suggest the full binding affinity of the original rodent 3G9 is retained in the chimpanzee version of 3G9. Accordingly, the results show that CDR grafting of rodent CDRs onto chimpanzee acceptor frameworks as described in the present invention retained the full binding avidity of the parental rodent antibody.

A BIAcore analysis (Pharmacia) was performed to determine the binding affinity for human EPOr of murine 3G9 and CP3G9. The interaction of CP3G9 as well as murine 3G9 with EPOr was characterized using a BIAcore 1000 biosensor. Descriptions of the instrumentation and the sensor surfaces are described in Brigham-Burke *et al.*, *Anal. Biochem.*, *205*:125-131 (1992).

CP3G9 was captured onto a sensor surface of immobilized protein A. The kinetic binding constants were determined by passing solutions of monomeric EPOr over the surface and monitoring binding versus time. The equilibrium dissociation constant for the interaction was then derived from the ratio of the kinetic constants. The parent murine 3G9 was captured onto a surface of protein A captured rabbit anti-mouse Fc specific polyclonal antibody. The kinetics and dissociation constant for the interaction with EPOr was determined as described above. All measurements were made in 10 mM sodium phosphate, 150 mM NaCl pH 7.2-3 mM EDTA and 0.005% Tween 20. The flow rate was 60 uL/min. The temperature was 20° C.

	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})	K_D (nM)
murine 3G9	$1.2x10^6$	$4.0x10^{-3}$	3.3
CP3G9	$1.0x10^6$	$9.1x10^{-3}$	9.1

These results show that the dissociation equilibrium constants determined for the murine and chimpanzee framework versions of 3G9 are within three fold of each other. This

data is in good agreement with the results of the ELISA-based study described above. Accordingly, the results show that the process used in generating the chimpanzee version of 3G9 largely retained the binding affinity of the original rodent mAb.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof, and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.